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PARTIAL DELETION OF TIE2 AFFECTS MICROVASCULAR ENDOTHELIAL RESPONSES TO CRITICAL ILLNESS IN A VASCULAR BED AND ORGAN-SPECIFIC WAY

Rianne M. Jongman,[*†‡] Peter J. Zwiers,[*] Bart van de Sluis,[§] Marleen van der Laan,[*] Jill Moser,[*†] Jan G. Zijlstra,[†] Daphne Dekker,[§] Nicolette Huijkman,[§] Henk E. Moorlag,[*] Eliane R. Popa,[*] Grietje Molema,[*] and Matijs van Meurs[*†]

[*]Department of Pathology and Medical Biology, Medical Biology Section, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; [†]Department of Critical Care, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; [‡]Department of Anesthesiology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; and [§]Department of Pediatrics, Molecular Genetics Section, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

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ABSTRACT—Tyrosine kinase receptor (Tie2) is mainly expressed by endothelial cells. In animal models mimicking critical illness, Tie2 levels in organs are temporarily reduced. Functional consequences of these reduced Tie2 levels on microvascular endothelial behavior are unknown. We investigated the effect of partial deletion of Tie2 on the inflammatory status of endothelial cells in different organs. Newly generated heterozygous Tie2 knockout mice (exon 9 deletion, ΔE9/Tie2$^{+/-}$) exhibiting 50% reduction in Tie2 mRNA and protein, and *wild-type* littermate controls (Tie2$^{+/+}$), were subjected to hemorrhagic shock and resuscitation (HS + R), or challenged with *i.p.* lipopolysaccharide (LPS). Kidney, liver, lung, heart, brain, and intestine were analyzed for mRNA levels of adhesion molecules E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular cell adhesion molecule 1 (ICAM-1), and CD45. Exposure to HS + R did not result in different expression responses of these molecules between organs from Tie2$^{+/-}$ or Tie2$^{+/+}$ mice and sham-operated mice. In contrast, the LPS-induced mRNA expression levels of E-selectin, VCAM-1, and ICAM-1, and CD45 in organs were attenuated in Tie2$^{+/-}$ mice when compared with Tie2$^{+/+}$ mice in kidney and liver, but not in the other organs studied. Furthermore, reduced expression of E-selectin and VCAM-1 protein, and reduced influx of CD45^{+} cells upon LPS exposure, was visible in a microvascular bed-specific pattern in kidney and liver of Tie2$^{+/-}$ mice compared with controls. In contrast to the hypothesis that a disbalance in the Ang/Tie2 system leads to increased microvascular inflammation, heterozygous deletion of Tie2 is associated with an organ-restricted, microvascular bed-specific attenuation of endothelial inflammatory response to LPS.

KEYWORDS—Adhesion molecules, endotoxemia, inflammation, leukocyte influx, microvascular endothelium, Tie2

ABBREVIATIONS—Ang(x)—Angiopoietin (x); HS+R—hemorrhagic shock followed by resuscitation; ICAM-1—intercellular adhesion molecule 1; LPS—lipopolysaccharide; NF-κB—nuclear factor-kappaB; Tie2—tyrosine-protein kinase receptor; Tie2$^{+/-}$—heterozygous Tie2 knockout mice; Tie2$^{+/+}$—*wild type* littermate controls; VCAM-1—vascular cell adhesion molecule 1; WT—wild type

INTRODUCTION

Tyrosine kinase receptor (Tie2) is mainly expressed by endothelial cells (1). Tie2 interacts with its ligands Angiopoietin (Ang) 1 and Ang2 to facilitate blood vessel development, and vessel stabilization or destabilization in mature vessels. In quiescent conditions of the mature vasculature, Ang1 binds to Tie2 leading to dimerization of the Tie2 receptor and subsequent activation of several intracellular pathways that maintain endothelial integrity (2).

In inflammatory conditions, the endothelium becomes activated and expresses adhesion molecules such as E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular cell adhesion molecule 1 (ICAM-1), which serve as guidance for leukocytes to move to the site of inflammation. Furthermore, Ang2 is secreted by activated endothelial cells to induce destabilization of the endothelium by competing with Ang1 for the Tie2 receptor, leading to increased vascular permeability (3). Data also suggest the existence of a functional link between the Angs and the response of endothelial cells in inflammation (4, 5). For example, adenoviral production of Ang1 inhibited in-vivo leukocyte infiltration in a lipopolysaccharide (LPS)-induced endotoxemia mouse model (6). Similarly, in-vitro Ang1 treatment partially inhibited adhesion and transendothelial migration of leukocytes, which was accompanied by suppressed expression

Address reprint requests to Grietje Molema, PhD, Department of Pathology and Medical Biology, Medical Biology Section, University Medical Center Groningen, University of Groningen, Hanzeplein 1, IPC EA11, 9713 GZ Groningen, The Netherlands. E-mail: g.molema@umcg.nl

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of adhesion molecules expression in endothelial cells (7, 8). In Ang2 knockout mice, reduced leukocyte influx of neutrophils in response to *i.p* injection of bacteria was reported (9). Moreover, in-vivo blockade of Ang2 reduced infiltration of leukocytes and expression of adhesion molecules in the lung, and at the same time inhibited vascular remodeling (10).

Although we understand the effects of changes in concentration of the ligands Ang1 and Ang2 in plasma of critically ill patients, we know little about the effects of changes in expression levels of the Tie2 receptor on the inflammatory response of the endothelium in organs. Previously, we reported reduced expression of Tie2 in kidney biopsies of sepsis patients (11), as well as in organs of mice subjected to hemorrhagic shock and LPS-induced endotoxemia (12). However, the functional consequences of this reduced expression for endothelial behavior were not explored. In the present study, our aim was to investigate the effects of partial deletion of Tie2 on endothelial responses in 2 animal models of critical illness, with focus on the microvasculature in different organs of these mice, as endothelial cells in different (micro)vascular beds were previously reported to respond differently to inflammatory stimuli (13–15).

To this end, we created a condition of lower Tie2 expression by generating a heterozygous Tie2 knockout mouse model based on deletion of exon 9 (ΔE9/Tie2$^{+/-}$, hereafter referred to as Tie2$^{+/-}$). We verified that these mice express 50% lower Tie2 protein compared with their *wild type* (WT) littermate controls, after which we investigated whether this genetically constructed reduction in Tie2 expression affected basal expression of the Tie2 ligands Ang1 and Ang2 and basal endothelial inflammatory genes. We further examined the effects of hemorrhagic shock followed by resuscitation (HS + R), and of endotoxemia induction by *i.p*. LPS treatment on endothelial responses and leukocyte recruitment to the organs. We compared responses in Tie2$^{+/-}$ mice with those in WT mice by studying whole organ responses as well as responses in specific microvascular segments in these organs.

MATERIALS AND METHODS

Generation of heterozygous Tie2$^{+/-}$ mice

The Tie2^{floxed} mouse line was generated by homologous recombination of the Tie2 allele using a method described previously (16). Briefly, a genomic fragment (12.2 kb) of the Tie2 gene spanning exons 9–11 was obtained from bacterial artificial chromosomes #bMQ279D1 (129S7/SvEv embryonic stem cell, Source BioScience, Nottingham, United Kingdom) and cloned into the pDTA.4B vector. An orphan loxP site was inserted into the pDTA.4B-Tie2 (exon9-exon11) construct, 119 bp upstream of exon 9 using recombineering (Fig. 1A). The *frt-neo-frt-loxP* cassette was inserted into the targeting construct 189 bp downstream of exon 9. The final construct was linearized with *Apa*I and electroporated into TL1 129Sv/E embryonic stem cells. Subsequently, the cells were selected in medium supplemented with G418, and expanded. Southern blot analysis was performed using a 198 bp 5′ external probe on *Eco*RI-digested genomic embryonic stem cell DNA (Fig. 1B). Oligosequences used for recombineering and the Southern blot probe can be obtained upon request.

Chimeric mice were generated by microinjection of 2 independent embryonic stem cell-targeted clones into C57BL/6 blastocysts. Chimeric males were mated with C57BL/6 females and germ line transmission of the floxed Tie2 allele (Tie2$^{floxed\text{-}neo}$) was confirmed by PCR analysis using 5′-GCTCGACGTTGTCACTGAAG-3′ and 5′-CCATTTTCCACCATGATATTCG-3′ primers. The neo cassette was excised by breeding the Tie2$^{floxed\text{-}neo}$ mice with mice expressing flippase recombinase (*ACTFLPe*, Jackson Laboratory, Bar Harbor, strain #005703).

Mice carrying 1 Tie2 null allele (Tie2$^{+/-}$) were generated by crossing Tie2$^{floxed/floxed}$ mice with mice expressing Cre-recombinase in the female germ line (*Hprt-Cre*, Jackson Laboratory, strain #004302). In this study, litters resulting from F_1 intercrossing of Tie2$^{+/-}$ mice were used.

Genotyping

Mouse genomic DNA was extracted from ear punches using standard protocols. The genotype of Tie2^{floxed} mice was determined by PCR analysis using 5′-GGGCTGCTACAATAGCTTTGG-3′ and 5′-GGCCACTGAGAAACGATCTG-3′ primers, resulting in a 338 bp PCR product when loxP sites were present (Tie2$^{floxed/+}$) and in a 218 bp PCR product when loxP sites were absent (Tie2$^{+/+}$; Fig. 1C).

The genotype of Tie2$^{+/-}$ mice was determined by PCR using the primers 5′-GGGCTGCTACAATAGCTTTGG-3′ and 5′-GTTATGTCCAGTGTCAATCAC-3′ resulting in a 644 bp PCR product when exon 9 is still present (Tie2$^{+/+}$) and in a 309 bp PCR product when exon 9 of Tie2 was excised by Cre-recombinase (Tie2$^{+/-}$; Fig. 1D). PCR products were run on a 1.5% (*w/v*) agarose gel in Tris-borate-EDTA-buffer with 0.005% (*v/v*) ethidium bromide, and visualized under UV light.

Mouse shock models

Hemorrhagic shock model—Mouse hemorrhagic shock was induced as previously described (17). Briefly, mice were anesthetized with isoflurane and kept on a temperature-controlled (37°C–38°C) surgical pad. Hemorrhagic shock was induced by blood withdrawal from the left femoral artery, until a reduction of the mean arterial pressure to 30 mmHg was reached. To maintain the mean arterial pressure at 30 mmHg, small volumes of blood were withdrawn or restituted during the shock period. After 90 min of shock, mice were resuscitated (HS + R) with 4% human albumin in saline (Sanquin, Amsterdam, The Netherlands) at two times the volume of blood withdrawn. Mice were sacrificed 1 hour after resuscitation, because our previous studies showed increased mRNA expression of endothelial adhesion molecules in mouse organs at 1 h after resuscitation after 90 min of hemorrhagic shock (13). Sham-operated mice underwent instrumentation and were kept under anesthesia for the same period as hemorrhagic shock mice, without withdrawal of blood. At sacrifice, blood was drawn via cardiac puncture and organs were harvested, snap-frozen on liquid nitrogen and stored at −80°C until analysis. Groups consisted of 6 mice each.

Endotoxemia model—To induce endotoxemia, mice were *i.p.* injected with 1 μg/g body weight LPS (*E. coli*, serotype O26:B6, Sigma-Aldrich, St. Louis, MO) in NaCl 0.9% (*w/v*). Vehicle control mice were injected *i.p.* with NaCl 0.9% (*w/v*). All mice were sacrificed under isoflurane/O_2 anesthesia 4 h after LPS or vehicle administration, because our previous studies showed increased mRNA expression of endothelial adhesion molecules in mouse organs after 4 h after LPS injection (18).

Blood was drawn via cardiac puncture and organs were harvested, snap-frozen on liquid nitrogen and stored at −80°C until analysis. Groups consisted of 6 mice each.

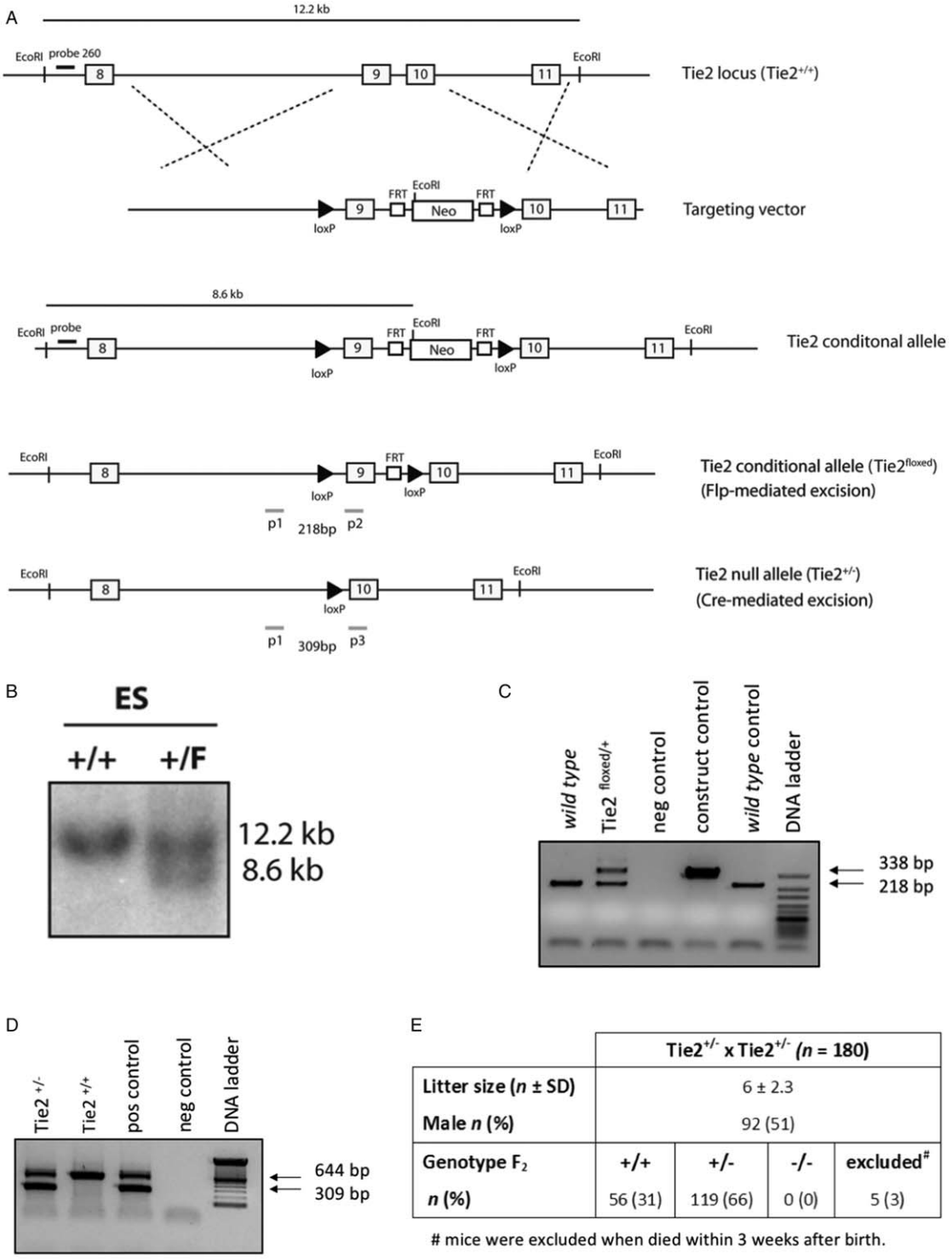
RNA isolation and gene expression analysis by quantitative RT-PCR

To study gene expression levels, total RNA was isolated from tissues with the RNeasy Plus Mini Kit, (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. RNA concentration (optical density $[OD]_{260}$) and purity (OD_{260}/OD_{280}) was measured using an ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Rockland, DE). RNA integrity was determined by gel electrophoresis.

cDNA was synthesized using random hexamer primers (Promega, Leiden, The Netherlands) and SuperScript III (Invitrogen, Breda, The Netherlands). Assay-on-demand primers/probe sets (TaqMan Gene Expression) were purchased from Thermo Fisher Scientific (Bleiswijk, The Netherlands) (Table 1). Duplicate quantitative PCR analyses were performed on ViiA7 real-time PCR system (Thermo Fisher Scientific) for each sample and the obtained threshold cycle values (CT) were averaged. Gene expression was normalized to the expression of the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*), yielding the ΔCT value. The average mRNA level relative to GAPDH was calculated by $2^{-\Delta CT}$.

Protein quantification by ELISA

Tissue homogenates were prepared from cryosections of organs by lysis in radioimmunoprecipitation assay buffer on ice (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% (*w/v*) sodium deoxycholate, 0.1% (*w/v*) SDS, 1% (*v/v*) IGEPAL® CA-630, Sigma-Aldrich, St. Louis, Mo) containing protease inhibitor (Roche Diagnostics, Almere, The Netherlands), phosphatase inhibitor (Roche), and 1 mM activated Na_3VO_4. Total protein concentration was determined by DC Protein Assay (Bio-Rad Laboratories, Veenendaal, The Netherlands).



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	Tie2$^{+/-}$ x Tie2$^{+/-}$ (n = 180)			
Litter size (n ± SD)	6 ± 2.3			
Male n (%)	92 (51)			
Genotype F_2	+/+	+/-	-/-	excluded#
n (%)	56 (31)	119 (66)	0 (0)	5 (3)

mice were excluded when died within 3 weeks after birth.

FIG. 1. **Generation of the Tie2^{floxed} mouse line.** (A) Schematic representation of the 12.2 kb genomic fragment of Tie2. LoxP sites were inserted up and downstream of exon 9. p1, p2, and p3 represent binding sites for primers resulting in PCR products as visualized in C and D. (B) Southern blot analysis using a 5′ external probe on *EcoR*I-digested gDNA. $^{+/+}$ *wild-type* allele; $^{+/F}$, floxed allele (C) Genomic PCR analyses with primer p1 and p2 confirmed presence (338 bp, Tie2$^{floxed/+}$) or absence of loxP sites (218 bp, WT). (D) Tie2$^{floxed/floxed}$ male offspring crossed with *Hprt-cre* females produced a cre-mediated excision and resulted in a 309 bp (Tie2$^{+/-}$) PCR product when exon 9 was excised and/or 644 bp (Tie2$^{+/+}$) PCR product when exon 9 was present using primers p1 and p3. (E) Characteristics and genotypes of the offspring of F_1 intercross Tie2$^{+/-}$ mice. WT, wild type.

Protein expression of Tie2 in organs was quantified by ELISA according to manufacturer's instructions (R&D Systems, Abingdon, UK). Tie2 amounts were normalized for the total protein input of tissue homogenate and expressed as pg/μg total protein. Protein concentration of soluble Tie2 was measured in plasma using the same ELISA kit.

Localization of proteins by immunohistochemistry

To study protein expression in different microvascular beds in organs, 4 μm cryosections were cut and fixed with acetone. After blocking endogenous peroxidase with 0.075% (*v/v*) H_2O_2 in PBS, sections were incubated for 1 h

TABLE 1. **RT-qPCR primers**

Gene	Assay ID	Encoded protein
Gapdh	Mm99999915_g1	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
Tek	Mm00443242_m1	Tyrosine kinase receptor (Tie2), CD202
Angpt1	Mm00456503_m1	Angiopoietin 1
Angpt2	Mm00545822_m1	Angiopoietin 2
Sele	Mm00441278_m1	E-selectin, CD62E
Vcam1	Mm00449197_m1	Vascular cell adhesion molecule 1 (VCAM-1), CD106
Icam1	Mm00516023_m1	Intercellular adhesion molecule 1 (ICAM-1), CD54
Ptprc	Mm00448463_m1	Protein tyrosine phosphatase receptor type C, CD45

GAPDH indicates Glyceraldehyde-3-phosphate dehydrogenase; Tie2, tyrosine kinase receptor; RT-qPCR, reverse transcription quantitative polymerase chain reaction; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule.

at room temperature with primary antibodies for E-selectin (clone Mes-1, a kind gift from Dr. Brown, UCB Celltech, Brussels, Belgium), VCAM-1 (clone M/K-2, Merck Millipore, Amsterdam, The Netherlands), or CD45 (clone 30-F11, BD Biosciences, Breda, The Netherlands). All primary antibodies were diluted in PBS 5% (*v/v*) fetal calf serum (Sigma-Aldrich). Isotype controls IgG1, IgG2a, and IgG2b (Antigenix America, New York, NY) were consistently found to be negative. Next, slides were incubated with secondary rabbit-anti-rat IgG antibody (Vector Laboratories, Burlingame, CA) in PBS supplemented with 5% (*v/v*) fetal calf serum and 1% (*v/v*) normal mouse serum (Sanquin) for 45 min, followed by anti-rabbit, horseradish peroxidase-labeled polymer (Dako Netherlands, Heverlee, Belgium) for 30 min. Between incubation steps, slides were washed extensively with PBS. Peroxidase activity was detected with 3-amino-9-ethylcarbazole (Sigma-Aldrich). Sections were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany).

Stained sections were scanned with NanoZoomer 2.0 HT (Hamamatsu Photonics, Almere, The Netherlands). Immunohistochemical stainings were quantified using Aperio Imagescope software v12.1 (Leica Biosystems Imaging, Vista, CA). Briefly, regions of interest were drawn around the perimeter of the tissue sections, excluding occasional artifacts (tissue breaks or folds). After automated counting of pixels, the ratio of positive pixels/total pixels was calculated. Next, the fold change of the ratio positive pixels/total pixels between LPS-challenged mice and their vehicle controls was calculated and plotted.

Statistical analysis

The proper control for HS + R is sham (instrumentation and anesthesia without withdrawal of blood), and not untreated mice, as sham itself induces endothelial inflammatory responses (13), for LPS it is vehicle control (*i.p* injection with NaCl 0.9%). We therefore compared gene expression levels between $Tie2^{+/-}$ mice and $Tie2^{+/+}$ mice exposed to HS + R or LPS using fold change of expression levels between HS + R and sham, respectively, LPS challenge and vehicle controls. This was calculated as follows: average relative mRNA expression of the sham or vehicle-treated $Tie2^{+/+}$ or $Tie2^{+/-}$ group was set at 1. Relative mRNA levels of individual HS + R or LPS-treated mice were divided by the average mRNA levels of their respective sham or vehicle group. Statistical significance between $Tie2^{+/+}$ and $Tie2^{+/-}$ mouse responses was evaluated by a two-tailed unpaired Student's *t* test. Statistics were performed using GraphPad Prism 7.0 (GraphPad Prism Software Inc. La Jolla, CA). Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Generation and characterization of $Tie2^{+/-}$ mice

We first constructed a $Tie2^{+/-}$ mouse line by the deletion of exon 9 of Tie2. Crossing homozygous $Tie2^{floxed/floxed}$ mice with *Hprt-Cre* mice resulted in 100% $Tie2^{+/-}$ offspring. F_1 intercrossing of $Tie2^{+/-}$ mice resulted in F_2 generations of which 66% were $Tie2^{+/-}$ mice, and 31% were $Tie2^{+/+}$. $Tie2^{-/-}$ mice were not born (Fig. 1E).

To confirm that Tie2 levels were indeed reduced by 50% in the newly generated mouse line, we analyzed Tie2 expression levels in kidney, liver, lung, heart, brain, and intestine. In these organs, Tie2 mRNA and protein levels were approximately 50% lower in $Tie2^{+/-}$ mice compared to $Tie2^{+/+}$ littermates (Fig. 2).

As Tie2 was reported to be expressed not only by endothelial cells, but also, to a minimal extent, by hematopoietic cells (19), we analyzed Tie2 mRNA expression in total white blood cell isolates of $Tie2^{+/+}$ and $Tie2^{+/-}$ mice. Tie2 mRNA was not detectable in white blood cells of either mouse line, in contrast to the highly expressed pan-leukocyte marker protein tyrosine phosphatase receptor type C (*Ptprc*) encoding CD45 protein (Suppl. Table 1, http://links.lww.com/SHK/A795). Thus, deletion of exon 9 of Tie2 from one allele resulted in a 50% reduction of Tie2 expression in the organs.

Basal mRNA expression levels of angiopoietins and genes related to endothelial inflammatory activation in $Tie2^{+/-}$ mice

As Tie2 is constitutively expressed by endothelial cells, a reduction in its protein levels, as affected by partial knockout of the Tie2 gene at the start of life in embryo, may potentially result in adaptation of expression of its ligands Ang1 and Ang2. We found no differences in basal mRNA expression levels of Ang1 and Ang2 in kidney, liver, lung, heart, brain, and intestine between $Tie2^{+/-}$ mice and $Tie2^{+/+}$ mice, irrespective of the organ studied (Suppl. Figure 1A, http://links.lww.com/SHK/A795).

Next, we examined whether partial deletion of Tie2 has consequences for basal expression levels of the endothelial inflammatory activation genes E-selectin, VCAM-1, and ICAM-1 (Figure S1B, http://links.lww.com/SHK/A795). In both mouse lines, basal expression of these genes showed organ-dependent differences. The highest expression of E-selectin, VCAM-1, and ICAM-1 was found in the lung, whereas the lowest expression of E-selectin and VCAM-1 was found in the brain, the lowest expression of ICAM-1 in intestine. No differences in basal gene expression were found between $Tie2^{+/-}$ and $Tie2^{+/+}$ mice in any of the organs. As the studied adhesion molecules are mainly expressed by endothelial cells, we also investigated the expression levels of endothelial-restricted molecules platelet endothelial cell adhesion molecule 1 (*Pecam1*, CD31) and vascular endothelial cadherin (*Cdh5*). These varied between organs because of the differences in endothelial content between organs, yet did not differ between $Tie2^{+/-}$ and $Tie2^{+/+}$ mice in any of the organs studied (Suppl. Figure 2, http://links.lww.com/SHK/A795).

The endothelial adhesion molecules, E-selectin, VCAM-1, and ICAM-1, participate in leukocyte adhesion and extravasation. As partial deletion of Tie2 protein did not affect basal expression of these molecules, we postulated that leukocyte recruitment for homeostatic surveillance purposes would also not be affected in quiescent organs. Indeed, mRNA of CD45 was detected in all organs and no differences between $Tie2^{+/-}$

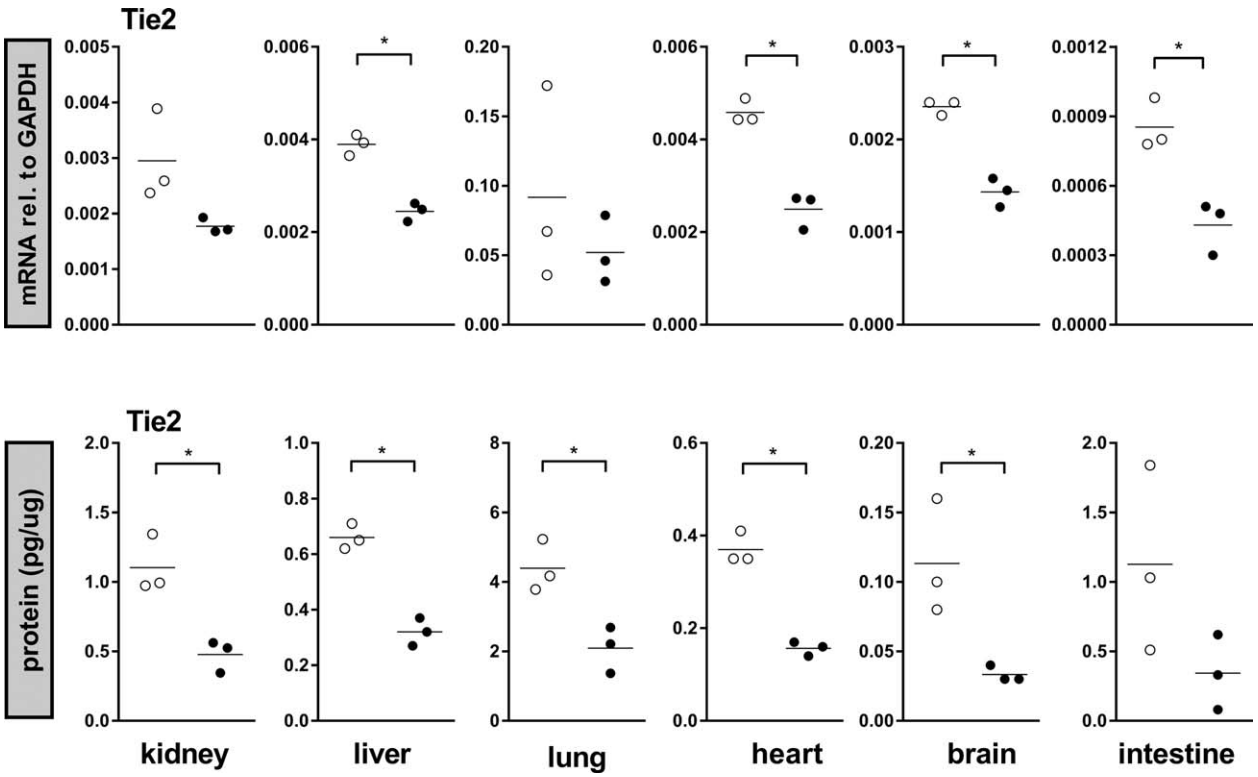

FIG. 2. **Tie2 expression is reduced to half in organs of Tie2$^{+/-}$ mice.** Organs of Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were assessed for mRNA and protein levels. (A) Tie2 mRNA levels determined by reverse transcription quantitative polymerase chain reaction relative to GAPDH. (B) Tie2 protein levels in organs determined by ELISA. Dots represent individual Tie2$^{+/+}$ mice (○), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 3 mice per group, $^{*}P < 0.05$ as evaluated with a two-tailed unpaired Student's *t* test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

and Tie2$^{+/+}$ mice were found in any of the organs analyzed (Suppl. Figure 1C, http://links.lww.com/SHK/A795).

In conclusion, reduction of Tie2 protein by 50% in Tie2$^{+/-}$ mice did not affect basal expression levels of its ligands Ang1 and Ang2. Moreover, basal expression levels of genes related to endothelial inflammatory activation, and the associated presence of leukocytes in the main organs, did not change.

Endothelial responses to hemorrhagic shock in Tie2$^{+/-}$ mice

We next investigated whether partial deletion of Tie2 protein affected endothelial responses in 2 models of critical illness. For this, we first employed HS + R, a model of critical illness which systemically affects all organs (13). We studied mRNA expression of endothelial adhesion molecules, E-selectin, VCAM-1, and ICAM-1, and CD45, in kidney, liver, and lung, as we have previously shown that these organs are most extensively affected by HS + R (17).

HS + R led to a reduction of Tie2 mRNA levels in kidneys of WT Tie2$^{+/+}$ mice, whereas in liver and lung in this experiment Tie2 mRNA levels were statistically not significantly different compared to sham controls (Suppl. Figure 3A, http://links.lww.com/SHK/A795). In Tie2$^{+/-}$ mice, in which Tie2 expression was already reduced by 50% at the start of hemorrhagic shock induction, the fold change of downregulation of Tie2 after HS + R in kidney, liver, and lung was similar as in WT mice (Suppl. Figure 3B, http://links.lww.com/SHK/A795).

The expression of E-selectin, VCAM-1, and ICAM-1 was not affected by HS + R in Tie2$^{+/+}$ or in Tie2$^{+/-}$ mice, irrespective of the organ (Fig. 3A). Moreover, CD45 mRNA levels did not differ between HS + R-treated mice and sham-treated mice in any of the organs of either genotype (Fig. 3B).

Summarizing, no changes in expression of genes related to endothelial activation and leukocyte influx could be observed between Tie2$^{+/-}$ and Tie2$^{+/+}$ mice when exposed to HS + R.

Endothelial responses to LPS in organs of Tie2$^{+/-}$ mice

As a second model of critical illness, we used LPS to induce endotoxemia to investigate whether partial deletion of Tie2 affected the expression of genes related to endothelial activation (20). We observed downregulation of Tie2 mRNA and protein in kidney, liver, lung, heart, brain, and intestine after LPS challenge in Tie2$^{+/+}$ mice, which confirmed previous data (12). In Tie2$^{+/-}$ mice, in which Tie2 expression was already reduced by 50% prior to LPS administration, Tie2 mRNA was additionally downregulated in all organs after LPS administration. The fold change downregulation of Tie2 mRNA was not different between Tie2$^{+/-}$ and Tie2$^{+/+}$ mice in any of the organs analyzed (Suppl. Figure 4, http://links.lww.com/SHK/A795).

Next, we studied the effect of LPS administration on the expression of the endothelial adhesion molecules. In all analyzed organs of both Tie2$^{+/-}$ and Tie2$^{+/+}$ mice, mRNA levels of E-selectin, VCAM-1, and ICAM-1 were increased after LPS administration compared with vehicle control (Fig. 4A and for

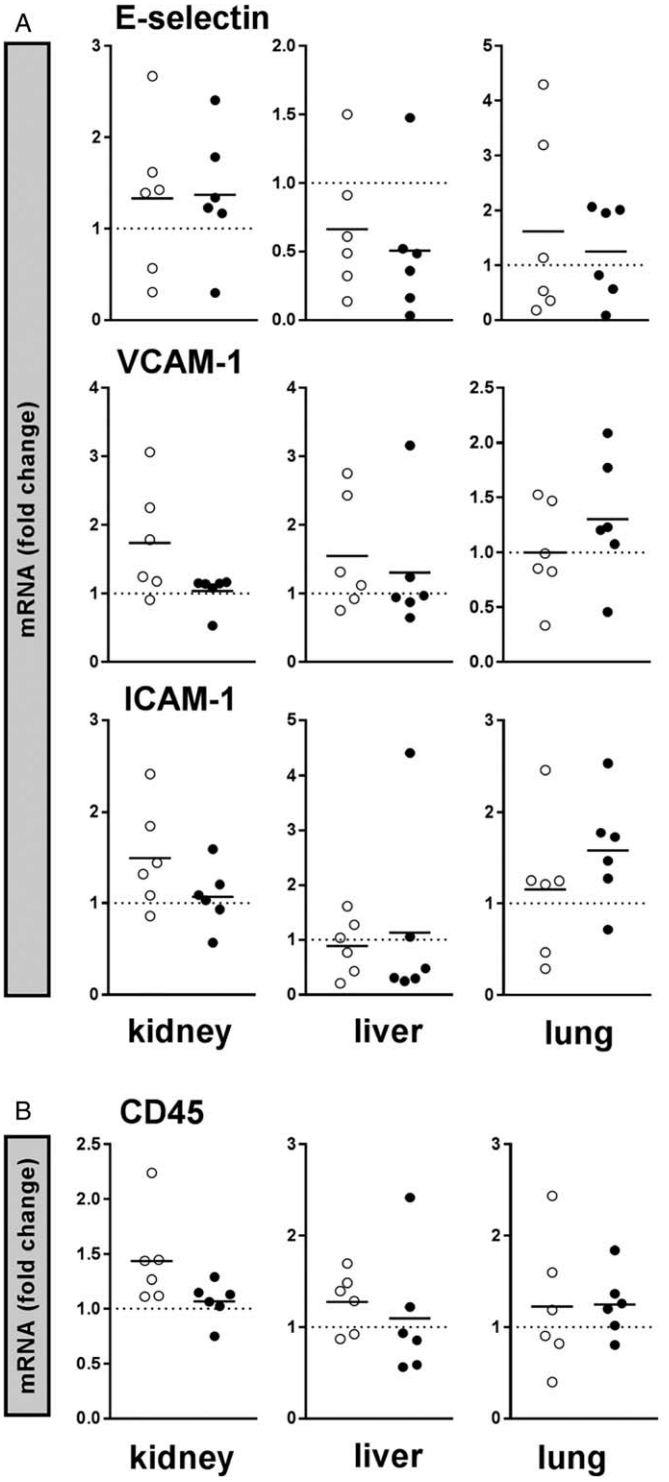

FIG. 3. **Expression of endothelial inflammatory responses to hemorrhagic shock and resuscitation in kidney, liver, and lung did not differ between Tie2$^{+/+}$ and Tie2$^{+/-}$ mice.** Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were subjected to HS + R and sacrificed 1 h after resuscitation. Organs were assessed for mRNA levels. (A) E-selectin, VCAM-1, and ICAM-1 mRNA levels. (B) CD45 mRNA levels. Data are presented as fold change between mice subjected to HS + R and sham (set at 1, —). Dots represent individual Tie2$^{+/+}$ mice (○), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 6 mice per group. Data are evaluated with a two-tailed unpaired Student's *t* test. HS + R, hemorrhagic shock and resuscitation; VCAM, vascular cell adhesion molecule.

VCAM-1 protein Supp. Figure 5A, http://links.lww.com/SHK/A795). Interestingly, the induction of expression of E-selectin and VCAM-1 was attenuated in kidney and liver, and of ICAM-1 in the liver, of LPS-treated Tie2$^{+/-}$ mice compared with Tie2$^{+/+}$ mice.

As we observed an attenuated induction of endothelial adhesion molecule expression in kidney and liver of Tie2$^{+/-}$ mice, we next investigated whether it affected leukocyte infiltration. mRNA expression of the leukocyte marker CD45 was increased in all organs after LPS exposure compared with vehicle control, irrespective of genotype (Fig. 4B and for MPO protein Suppl. Figure 5B, http://links.lww.com/SHK/A795). However, in LPS-challenged Tie2$^{+/-}$ mice, CD45 mRNA expression was also attenuated compared to its levels in Tie2$^{+/+}$ mice. This effect that was restricted to the kidney.

In summary, 50% reduction in Tie2 protein expression prior to challenge with LPS diminishes upregulation of inflammatory microvascular endothelial responses in an organ-specific way.

Microvascular bed-specific responses to LPS in Tie2$^{+/-}$ mice

After observing lower adhesion molecule expression in kidney and liver of Tie2$^{+/-}$ mice, we asked the question whether the diminished endothelial inflammatory response to LPS was associated with specific microvascular beds. To this end, we immunohistochemically detected E-selectin and VCAM-1 protein in kidney and liver sections of both mouse lines.

In kidney and liver of untreated mice of either genotype, E-selectin protein was not expressed in any microvascular segment (data not shown). After LPS exposure, E-selectin expression was visible in all microvascular beds in the kidney of both Tie2$^{+/-}$ and Tie2$^{+/+}$ mice, with highest expression in glomeruli and lowest in the peritubular capillaries (Fig. 5A). Planimetric quantification revealed no differences in E-selectin protein expression in the different microvascular beds of the kidney between Tie2$^{+/-}$ and Tie2$^{+/+}$ mice (Fig. 5A, lower panel). In the liver of both groups, strong E-selectin expression was observed in the sinusoidal capillaries and the venules in response to LPS challenge (Fig. 5B). In sinusoidal capillaries of Tie2$^{+/-}$ mice, E-selectin expression was diminished compared with its levels in their littermate controls. Planimetric quantification of the liver was restricted to total liver, and revealed diminished expression of E-selectin in heterozygous Tie2$^{+/-}$ mice compared with WT Tie2$^{+/+}$ mice.

In untreated mice, VCAM-1 was expressed in all microvascular beds in both kidney and liver. In the kidney, the highest VCAM-1 expression was observed in arterioles, and the lowest expression in glomeruli. In the liver, the extent of VCAM-1 expression was similar in sinusoidal capillaries and venules as microscopically assessed by eye (data not shown). In the kidney of Tie2$^{+/+}$ mice, LPS exposure elicited increased VCAM-1 expression in glomeruli, peritubular capillaries, and venules, whereas in arterioles its expression remained high (Fig. 5C). Possibly, additionally induced expression in this particular microvascular segment was masked by already high expression under control conditions. In Tie2$^{+/-}$ mice, LPS treatment led to increased VCAM-1 expression as well, yet the extent of expression in glomeruli, peritubular capillaries, and venules was lower compared with that in Tie2$^{+/+}$ mice. This was confirmed by planimetric analysis (Fig. 5C, lower panel). In

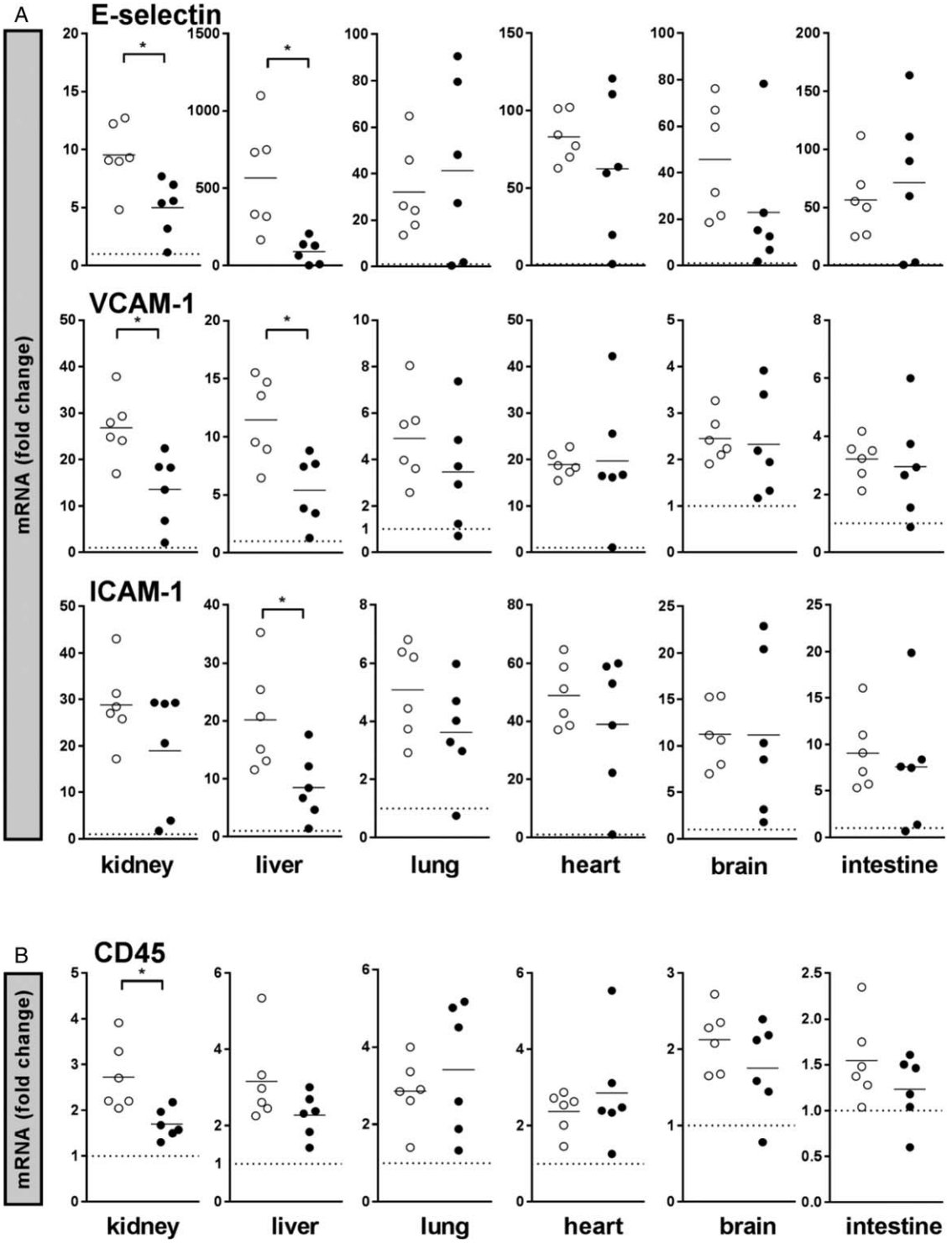

FIG. 4. **Tie2$^{+/-}$ mice showed diminished inflammatory responses of endothelial cells in distinct organs in response to LPS challenge.** Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were challenged with LPS *i.p.* (1 μg/g) and sacrificed 4 h later. Organs were assessed for mRNA. (A) E-selectin, VCAM-1, and ICAM-1 mRNA levels. (B) CD45 mRNA levels. Data are presented as fold change between LPS-treated mice and vehicle control (set at 1, —). Dots represent individual Tie2$^{+/+}$ mice (○), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 6 mice per group, $^{*}P < 0.05$ as evaluated with a two-tailed unpaired Student's *t* test. ICAM indicates intercellular adhesion molecule; LPS, lipopolysaccharide; VCAM, vascular cell adhesion molecule.

the liver, LPS-induced expression of VCAM-1 was observed in sinusoidal capillaries and in venules in both Tie2$^{+/-}$ and Tie2$^{+/+}$ mice (Fig. 5D). Compared with Tie2$^{+/+}$ mice, lower VCAM-1 expression was mostly observed in the sinusoidal capillaries of Tie2$^{+/-}$ mice. Planimetric analysis of total liver revealed lower VCAM-1 expression in Tie2$^{+/-}$ mice compared with Tie2$^{+/+}$ mice (Fig. 5D, lower panel).

To summarize, in kidney and liver pre-existent lower Tie2 levels in the Tie2$^{+/-}$ mice were associated with attenuated microvascular bed-specific expression of E-selectin and

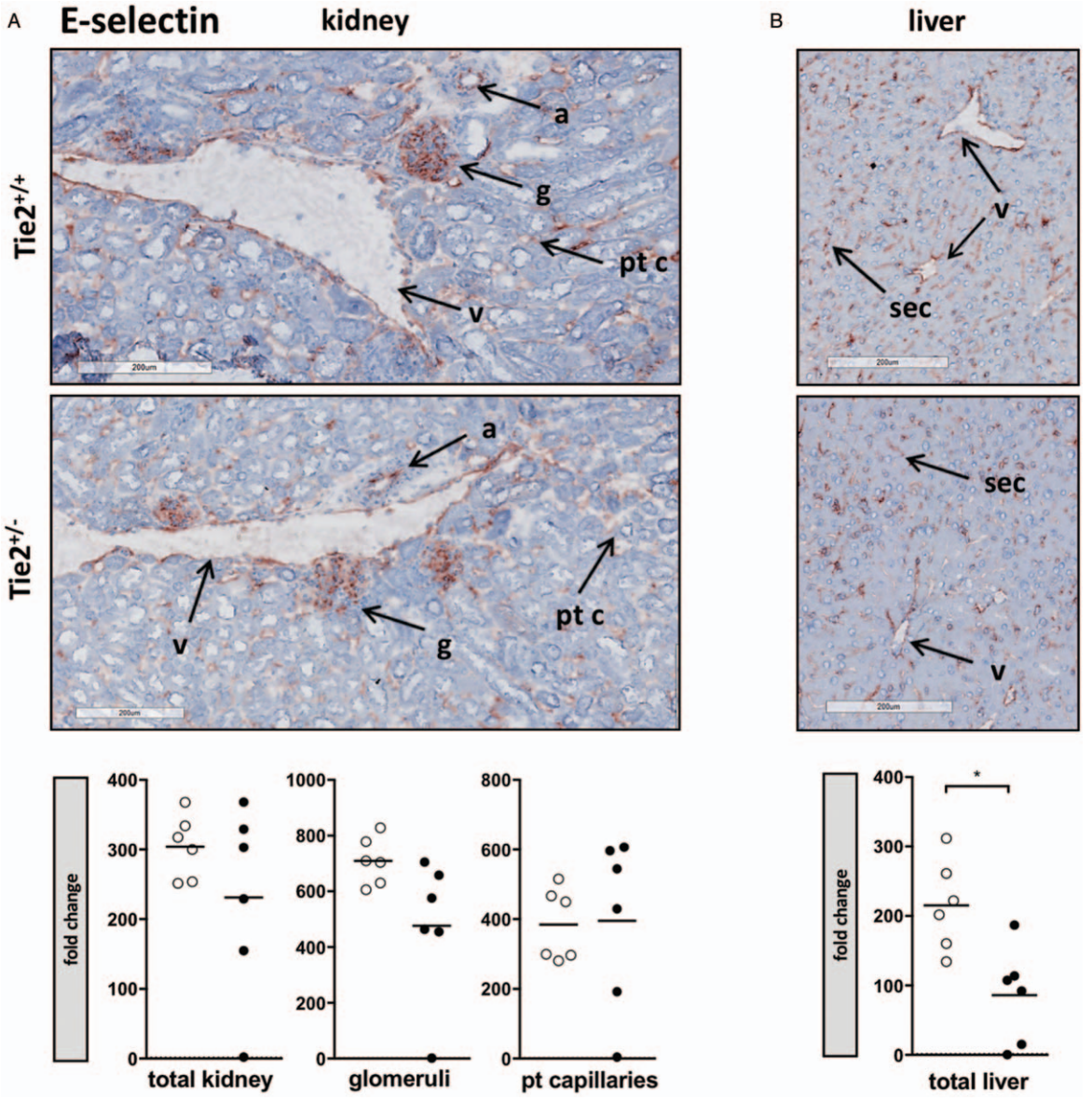

FIG. 5. **Tie2$^{+/-}$ mice showed diminished inflammatory responses of endothelial cells in an organ and microvascular bed-specific way in response to LPS challenge.** Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were challenged with LPS *i.p.* (1 μg/g) and sacrificed 4 h later. Organs were assessed for protein expression by immunohistochemistry. (A and B) Photomicrographs of cryosections of kidney (A) and liver (B) stained for E-selectin, and semiquantitative analysis of E-selectin expression in different microvascular segments by digital planimetry. (C and D) Photomicrographs of cryosections of kidney (C) and liver (D) stained for VCAM-1, and semiquantitative analysis of VCAM-1 expression in different microvascular segments by digital planimetry. Arrows indicate arterioles (a), glomeruli (g), peritubular capillaries (pt c), venules (v), and sinusoidal capillaries (sec). Scale bars 200 μm. Data are presented as fold change between LPS-treated mice and vehicle control (set at 1, —). Dots represent individual Tie2$^{+/+}$ mice (○), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 6 mice per group, $^{*}P < 0.05$ as evaluated with a two-tailed unpaired Student's *t* test. LPS indicates lipopolysaccharide; VCAM, vascular cell adhesion molecule.

VCAM-1 after LPS exposure, implying a role for Tie2 in regulating endothelial cell responses depending on the location of the endothelial cell in the organ.

Location of leukocyte influx in kidney and liver in response to LPS in Tie2$^{+/-}$ mice

As endothelial inflammatory adhesion molecules have a prominent role in leukocyte recruitment, we next investigated the effects of diminished expression on localization of infiltrating CD45^{+} leukocytes in kidney and liver of the WT and heterozygous Tie2$^{+/-}$ mice after LPS challenge. In control Tie2$^{+/+}$ and Tie2$^{+/-}$ mice, the many CD45^{+} cells were localized in renal peritubular capillaries, whereas some were visible in glomeruli (data not shown). After LPS administration, increased numbers of CD45^{+} cells localized in glomeruli and in the peritubular capillaries of the kidney in both Tie2$^{+/-}$ and Tie2$^{+/+}$ mice compared with vehicle controls (Fig. 6A). Compared with WT mice, lower numbers of CD45^{+} cells were observed in the renal peritubular capillaries in Tie2$^{+/-}$ mice. Planimetric quantification supported this observation (Fig. 6A, lower panel). In the liver of control Tie2$^{++/++}$ and Tie2$^{++/-}$ mice, scattered CD45^{++} cells

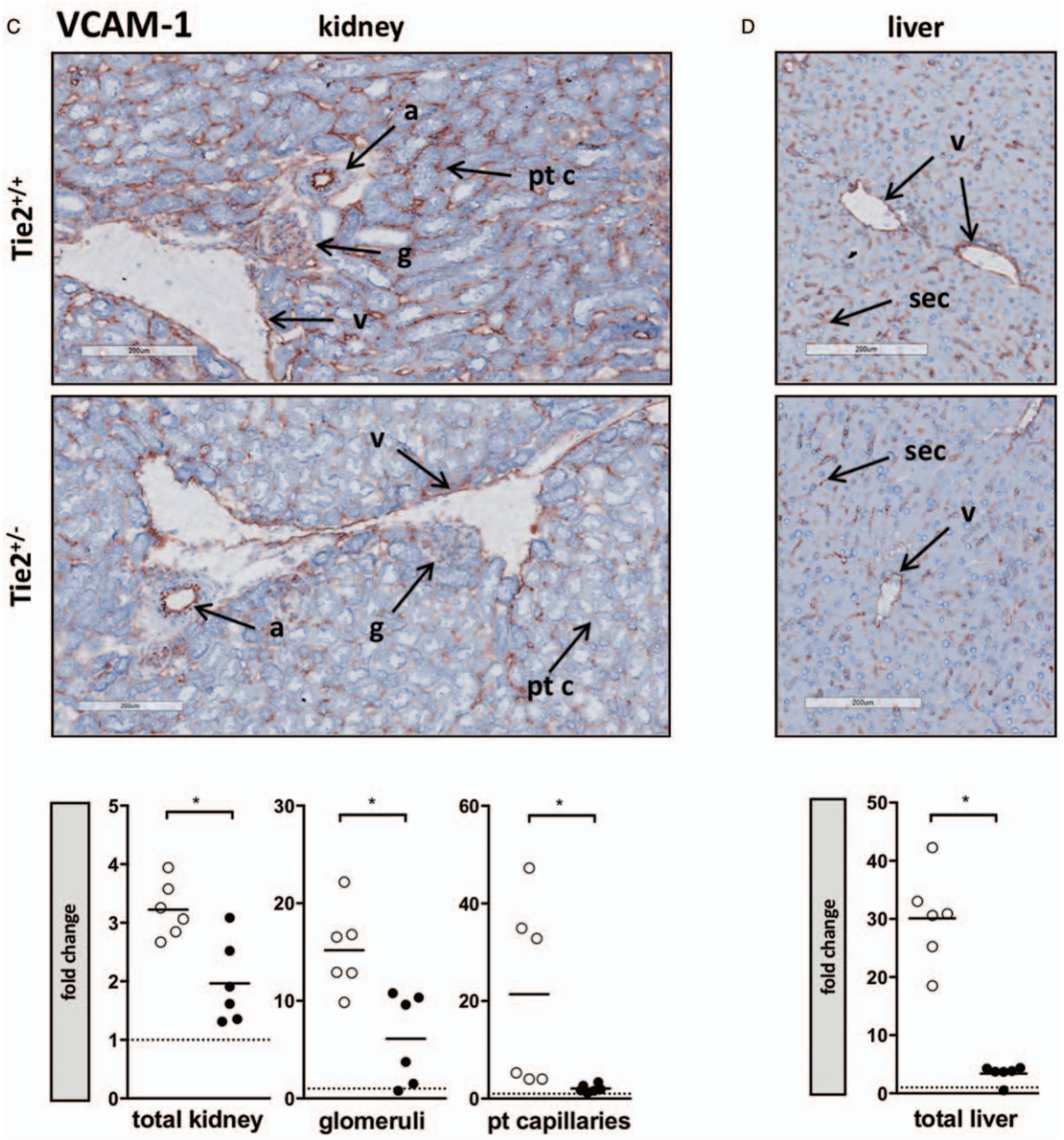

FIG. 5. (*Continued*)

were localized mainly in sinusoidal capillaries (data not shown). After LPS exposure, increased numbers of leukocytes were observed in sinusoidal capillaries in both Tie2$^{+/-}$ and Tie2$^{+/+}$ mice (Fig. 6B). Reduced numbers of CD45^{+} cells had accumulated in the sinusoidal capillaries of Tie2$^{+/-}$ mice compared with Tie2$^{+/+}$ mice. Planimetric quantification of the total liver confirmed reduced CD45^{+} cell localization in the liver of Tie2$^{+/-}$ mice compared with littermate controls (Fig. 6B, lower panel).

To summarize, lower Tie2 levels as present in Tie2$^{+/-}$ mice were associated with reduced numbers of leukocytes infiltrating in kidney and liver after LPS exposure, which is likely a consequence of the attenuated local expression of endothelial inflammatory adhesion molecules.

DISCUSSION

Tie2 is a tyrosine kinase receptor that is mainly expressed by blood vessel endothelial cells and plays a role in vascular integrity and inflammatory responses. Tie2 mRNA and protein levels are decreased in models of critical illness (12, 21). Although its ligands Ang1 and Ang2 have been extensively studied with regard to their spatiotemporal changes in expression and functional consequences thereof in response to inflammatory processes, functional consequences of reduced Tie2 levels on endothelial inflammatory responses in the microvasculature in organs are unknown. This study was designed to investigate effects of reduced Tie2 presence on the inflammatory responses of endothelial cells in the microvasculature in

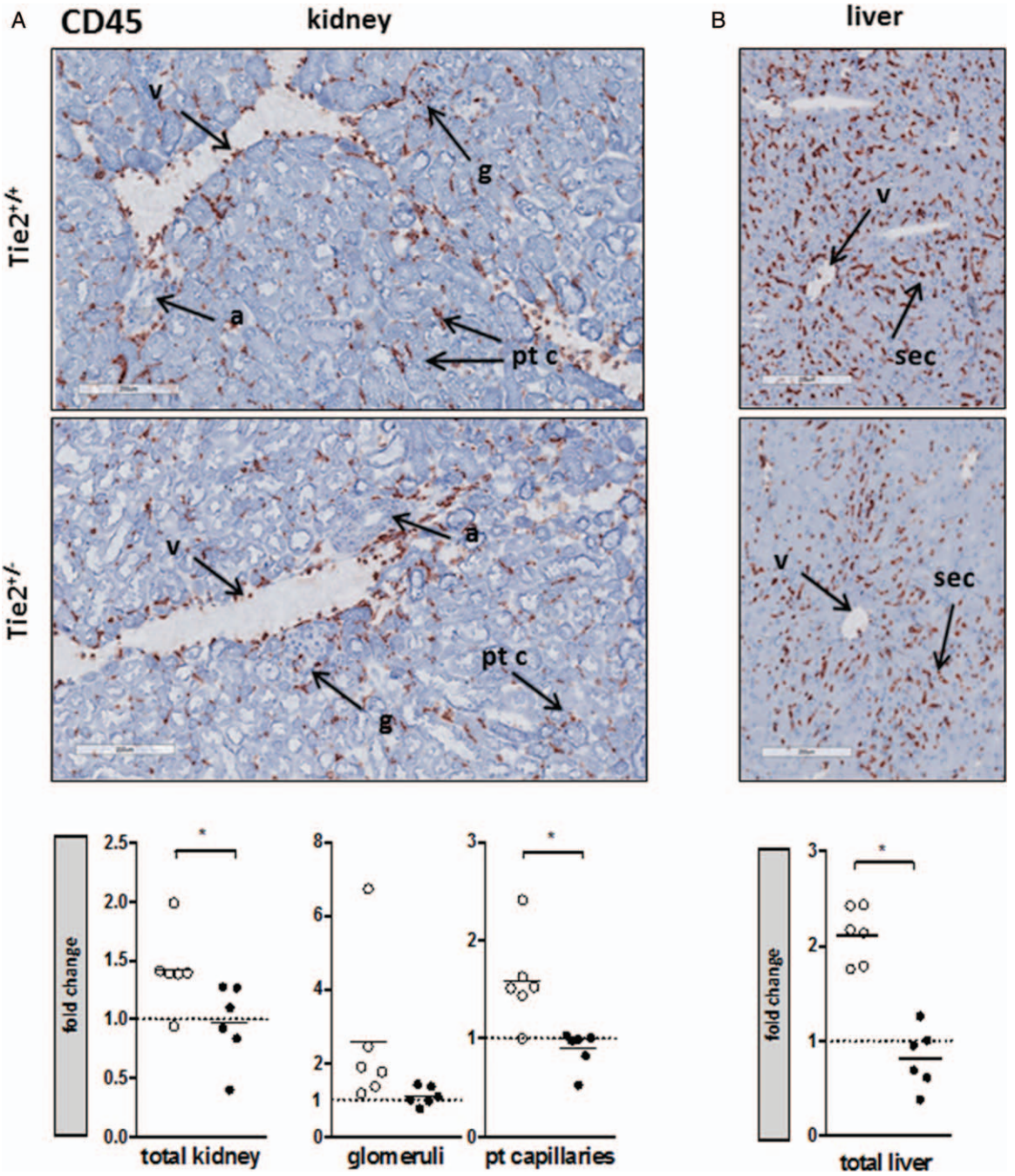

Fig. 6. **Tie2$^{+/-}$ mice showed diminished leukocyte influx in kidney and liver in response to LPS challenge.** Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were challenged with LPS *i.p.* (1 μg/g) and sacrificed 4 h later. Organs were assessed for protein expression by immunohistochemistry. (A and B) Photomicrographs of cryosections of kidney (A) and liver (B) stained for CD45^{+} leukocytes, and semiquantitative analysis of CD45^{+} cells in different microvascular segments by digital planimetry. Arrows indicate positive (red) cells in microvascular structures; arterioles (a), glomeruli (g), peritubular capillaries (pt c), venules (v), and sinusoidal capillaries (sec). Scale bars 200 μm. Data are presented as fold change between LPS-treated mice and vehicle control (set at 1, —). Dots represent individual Tie2$^{+/+}$ mice (○), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 6 mice per group, $^{*}P < 0.05$ as evaluated with a two-tailed unpaired Student's *t* test. LPS indicates lipopolysaccharide.

organs of mice in models of critical illness. In a newly generated heterozygous Tie2$^{+/-}$ mouse model in which deletion of exon 9 in one allele of the Tie2 gene resulted in 50% reduction of Tie2 expression, we showed that this loss did not affect basal expression levels of the Tie2 ligands Ang1 and Ang2, nor of endothelial inflammatory genes E-selectin, VCAM-1, and ICAM-1. We did not observe differences in inflammatory gene expression related to endothelial activation and leukocyte influx between Tie2$^{+/+}$ and Tie2$^{+/-}$ mice exposed to HS + R. LPS exposure on the other hand revealed an attenuated endothelial inflammatory response in mice expressing 50% less Tie2. This attenuated inflammatory response was restricted to the microvasculature of kidney and liver, and were shown to be microvascular bed and gene-specific.

Our new Tie2 mutant mouse line based on exon 9 deletion corroborates several findings in a previous Tie2 mutant mouse, generated by Dumont *et al.* (22), in which exon 1 of the Tie2 gene was deleted (ΔE1/Tie2$^{+/-}$). First, no homozygous Tie2 knockout mice were born in our Tie2 mutant line, which is in agreement with Dumont *et al.*'s observation that Tie2 homozygous knockout mice had embryonically lethal vascular malformations (22). Second, deletion of Tie2 in one allele in our model did not affect basal expression of the Tie2 ligands Ang1 and Ang2, nor that of endothelial adhesion molecules in any of the 5 organs studied. This complements previous data published by Ghosh *et al.* (23), using the aforementioned ΔE1/Tie2$^{+/-}$ mice and showed similar results on Ang1 and Ang2 expression in the lungs of Tie2$^{+/-}$ mice. Although Ghosh *et al.* focused solely on lung, our study is the first to report no changes in basal Ang1 and Ang2 expression levels in multiple individual organs of adult heterozygous Tie2$^{+/-}$ mice while experiencing lower Tie2 expression levels starting as early as in embryo. This indicates that adaptation to normalized expression levels of Ang1 and Ang2 to the lower Tie2 levels is not required for maintenance of vascular integrity in the adult microvasculature.

As previously reported, LPS administration suppresses Tie2 expression (12) and at the same time it induces activation of the nuclear factor-κB (NF-κB) pathway, leading to a proinflammatory endothelial response in mouse organs (8, 24). Our data on the absence of effects of lower Tie2 expression on endothelial inflammatory cell reaction to LPS in the lung support the findings by Ghosh *et al*. (23), who also did not observe differences in adhesion molecule expression in the lung of Tie2$^{+/-}$ mice that received 15 μg/g *i.p.* LPS when compared with WT controls. In contrast, McCarter *et al.* (25) reported in ΔE1/Tie2$^{+/-}$ mouse model reduced expression of E-selectin and VCAM-1 protein in lung compared with controls after intratracheal instillation of LPS at 800 μg dose. A possible explanation for the discrepancy between McCarter *et al.*'s (25) findings and those of Ghosh *et al.* (23) and ours could be that intratracheal instillation of LPS leads to higher local LPS levels than when administered *i.p.* Whether higher *i.p.* or intratracheally applied doses of LPS administered to our Tie2$^{+/-}$ mice would unmask Tie2 expression-related differences in adhesion molecule expression in lung needs to be established.

An important finding in our study is that a 50% reduction in Tie2 protein has functional consequences for particular microvessels in the body, whereas not affecting others. The molecular mechanism(s) behind this phenomenon is (are) unclear at present. Using laser dissection microscopy to isolate microvascular segments from kidneys of mice (26) prior to gene expression analysis, we found that each microvascular segment has its own Ang1/Ang2/Tie2 expression profile (unpublished data). Similarly, other endothelial cell controlling molecular systems such as vascular endothelial growth factor and its receptors are heterogenically expressed in the renal microvascular segments (21). How this links to the microvascular segment-specific responses to LPS in the absence of Tie2 as shown here remains elusive.

Microvascular endothelial cells play an important role in the development of multiple organ failure in patients treated on ICU units. The endothelial content and microenvironment (e.g., support cells, blood flow) differs per organ. Compared with the highly vascularized lung, the brain has relatively low endothelial content (Suppl Figure 2, http://links.lww.com/SHK/A795). As Tie2 is mostly expressed on endothelial cells, it is likely that these factors can affect the expression of Tie2 in the distinct organs and their response to stimuli (12). It is known that after LPS administration, Ang2 is released from endothelial Weibel–Palade bodies (3, 27) and can then compete with Ang1 for binding to Tie2, thereby inhibiting Tie2 phosphorylation (28). As a consequence, the NF-κB pathway is inhibited (29) and expression of proinflammatory genes is suppressed. Our results suggest that lowering Tie2 might be part of a feedback loop in reducing the inflammatory response. Studying the phosphorylation status of Tie2 in the different organs and microvascular segments of Tie2$^{+/-}$ mice as well as NF-κB nuclear translocation in time in response to LPS in both WT and Tie2$^{+/-}$ mice could shed light on this.

The dependence of endothelial cell responses to an inflammatory stimulus on Tie2 in particular microvascular beds were only observed in the endotoxemia model, not in the HS + R model. In this latter model, we observed a wide variation in microvascular responses in the HS + R groups as well as in sham groups of both genotypes. We did not perform a power analysis before starting animal experiments as we did not have an idea what the effect size would be as no data for the organs studied here has been reported before in this model or in a comparable model. As such, the results reported here could serve as a power analysis for future studies using this novel mouse line. It is of note that we used human albumin 4% as a resuscitation fluid, as 6% hydroxyethyl starch was withdrawn from our clinical arsenal because it increased the risk of renal dysfunction. Instead, a human colloid solution was used. However, it was recently demonstrated that the microvascular response of rats resuscitated with crystalloid and colloid infusions after hemorrhagic shock differ (30) and that the choice of resuscitation fluid influences neutrophil activation and soluble plasma levels of endothelial adhesion molecules in human trauma patients (31). Furthermore, fluid resuscitation with early blood-based regimes is tested in clinical care of HS + R patients. For future studies, it would therefore be of interest to study endothelial behavior in organs using other fluid resuscitation regimens in HS + R. Furthermore, the installation of the anesthesia and instrumentation procedure by itself already induces inflammatory responses (13) and is a confounding factor that may hamper identification of small differences between WT and transgenic mice in this critical illness model. We did not measure organ function, or blood gas (metabolic acidosis), and lactate levels in our mice to study clinically relevant organ failure parameters as our aim in this study was to focus on endothelial activation in organs. Finally, resuscitation in the LPS model would simulate the clinical situation better, as fluid resuscitation is a cornerstone of clinical sepsis treatment. However, we aimed to study a pure effect of LPS in these animals, as resuscitation itself varies in its effects on sepsis-induced neutrophil–endothelial cell interactions (32). Previously, a correlation between organ failure and soluble levels of the Ang/Tie2 system in plasma of ICU patients has been shown (33). Drugs aiming to restore the balance of the Angs in mouse

models of critical illness have shown to improve organ damage (34, 35), indicating a role for the Ang/Tie2 system in the development of organ failure. Tie2 signaling is important for the barrier function and thrombosis of microvessels (36), as well as the inflammatory status of the endothelial cells. As we have demonstrated that the receptor Tie2 is also a dynamic player in critical illness (11, 21), therapeutic intervention should not solely focus on the ligands Ang1 and Ang2, but should also focus on the Tie2 receptor.

Understanding the functional consequences of reduced levels of Tie2 in organs as observed in critical illness, may help to find or develop drugs to counteract the development of organ failure in the critical ill patient.

CONCLUSION

We here demonstrate that deletion of exon 9 in one allele of the Tie2 gene results in 50% less Tie2 expression. In contrast to the hypothesis that a disbalance in the Ang/Tie2 system leads to increased microvascular inflammation, the partial deletion of Tie2 had no significant effect on microvascular responses to HS + R as a model of critical illness, whereas after LPS administration lower Tie2 expression was associated with reduced endothelial responses in kidney and liver. These responses were restricted to particular microvascular beds in these organs, and were paralleled by changes in leukocyte recruitment. These data indicate that Tie2 has different functions in controlling endothelial cell behavior depending on the location and the microenvironment of the organ in the human body.

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